Antimicrobial Activity of Formylchromones: Detection by a Micro-Scale Method

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We report the antimicrobial activity of formylchromones. These compounds are remote structural analogues of nalidixic acid and quinolone antibiotics, and their activity was investigated by a simple micro-scale method designed for the determination of minimal inhibitory concentrations (MIC) of drug candidates and antibiotics against aerobic bacteria and yeasts. Minimal bactericidal and fungicidal concentrations (MBC and MFC, respectively) were also determined in connection with the MIC determinations. The results obtained were compared with those obtained using classical agar diffusion methodology. In the MIC method, deep-well micro-titration plates are used, covered by silicone sealing mats that allow diffusion of oxygen to the wells. The appropriate broth is pipetted into the wells, followed by a standardized microbial suspension (except for sterile controls) and a dilution series of the test substance or control antibiotic or a mere control solvent. The use of white non-transparent polypropylene plates allows easy visual inspection of microbial growth. For the MBC and MFC methods, samples are taken from all wells that contain a test substance or control antibiotic and do not display growth in the MIC test. The samples are streaked on agar plates, the liquid is allowed to absorb into the agar, and finally the microbes are spread all over the plate with a bent rod. Colony counts are compared with that of the untreated microbial suspension at the beginning of the MIC test. The MIC method is suitable for high-throughput screening.

Key words: Minimal Inhibitory Concentration, Minimal Bactericidal Concentration, Minimal Fungicidal Concentration, High-Throughput Screening Methods

Introduction

We have studied the antimicrobial activity of formylchromones **1a**–**e**. These compounds were chosen for study as drug candidates because their structure slightly resembles that of nalidixic acid (**2**), the parent structure of the quinolone antibiotics **3a** and **3b** (see Fig. 1 for structural formulas). Formylchromones are of interest as candidate antimicrobial agents also because they contain the aldehyde function (the formyl group). Many aldehydes are known to have highly potent antimicrobial activity (Bougault *et al.*, 1949; Burton *et al.*, 1964, 1965; Clarke *et al.*, 1963; Cronenberger *et al.*, 1968a, b, 1969; Rehn *et al.*, 1981; Taillandier and Pera, 1991; Pelttari *et al.*, 2007a, 2011).

In the discovery and development of antimicrobial drugs, agar diffusion methods (see, for example, Elo *et al.*, 2007; Pelttari *et al.*, 2002, 2007a, b; Rex *et al.*, 2009; Wikler *et al.*, 2009a) have retained a prominent position, since they are rapid to perform and are not as labour-intensive as classical

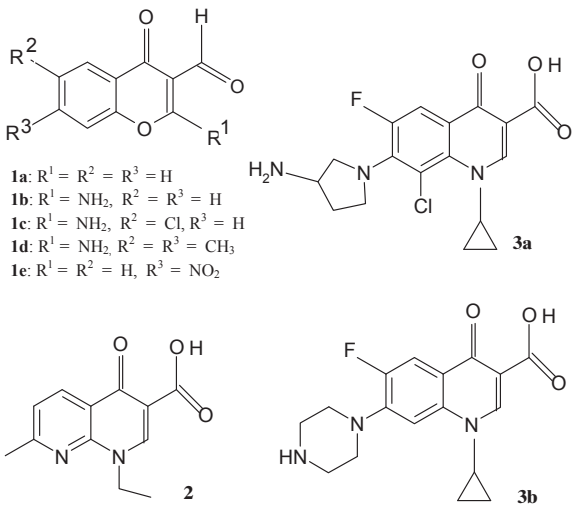

Fig. 1. Chemical structures of the 3-formylchromones **1a**–**e** studied, of nalidixic acid (**2**), and of two fluoroquinolone antibiotics, clinafloxacin (**3a**) and ciprofloxacin (**3b**).

macro-scale methods for determining the lowest concentration of the test substance capable of inhibiting the growth of a micro-organism [*i.e.*, the minimal inhibitory concentration (MIC)]. Especially if testing of a large number of substances against a large panel of microbial species or strains is to be performed, diffusion methods are the natural choice if the alternative is constituted by the determination of MIC values by classical macro-scale methods.

Antimicrobial susceptibility testing is important not only in the development and discovery of novel drugs but likewise also in diagnostic microbiology where it is used for determining treatment regimens for patients (Peterson and Shanholtzer, 1992). In clinical microbiology, the antibiotic susceptibility of a microbial strain is in some cases studied by determining its MIC value (Rex *et al.*, 2008a, b; Wikler *et al.*, 2009b).

Concerning possible clinical applications of drug candidates and, in clinical practice, the suitability of a specific antibiotic substance for the treatment of patients, it is important to verify that the substances are active essentially against all microbial cells of each susceptible strain. If a (small) portion of the cells of a microbial strain/culture is resistant to the test compound, it can be speculated that this might result in visible growth in a liquid culture but perhaps only in sporadic individual colonies on agar.

In determining the practical utility of an antimicrobial drug candidate, one further important parameter is constituted by its minimal microbicidal concentration, also called the minimal lethal concentration (MLC), *i.e.* the lowest concentration capable of killing the microbe in question, the most common examples of MLC being constituted by the minimal bactericidal and minimal fungicidal concentrations (MBC and MFC, respectively). The situation is the same in the clinical setting (Anhalt *et al.*, 1980; Peterson and Shanholtzer, 1992; Hacek *et al.*, 1999).

Thus, simple and rapid methods of determining MIC, MBC, and MFC values of antibiotics and drug candidates are needed. Considering drug discovery and development, such methods should preferably be suitable also for high-throughput screening. We report here such a simple and rapid micro-scale method that can be used for determining the MIC, MBC, and MFC values of test substances. The method is based on the use of deep-well micro-titration plates and, in this study, the method was tested using a panel of seven microbial species, including both bacteria and yeasts.

Experimental

Compounds tested

A total of five formylchromones (Fig. 1) were tested. They were obtained from Aldrich-Chemie/Aldrich Chemical Company (Steinheim, Germany). Compounds **1a** and **1e** were dissolved in dimethyl sulfoxide (DMSO). Compounds **1b**–**1d** were used as suspensions in DMSO, since no suitable solvent was found for them.

Microbial strains and culture conditions

A total of seven microbial strains were employed in this study (*Bacillus cereus*, *Candida albicans*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae*, *Staphylococcus aureus*, and *Streptococcus lactis*). The microbial strains (with the exception of *S. lactis*), their origins, the media employed, and the growth temperatures used have been previously described (Pelttari *et al.*, 2002, 2007a, b; Elo, 2007; Elo *et al.*, 2007). The antibiotics used as positive controls were: doxycycline (Doximycin; Orion, Espoo, Finland) for *B. cereus* and *P. aeruginosa*, cefuroxime (Zinacef; Glaxo Operations UK Ltd, Barnard Castle, UK) for *E. coli*, *S. aureus*, and *S. lactis*, and amphotericin B (Fungizone; Bristol-Myers Squibb, Bromma, Sweden) for the yeasts.

Agrar diffusion studies

All test compounds **1a**–**1e** were first screened employing the method described by Pelttari *et al.* (2002) and using the concentration 40 mg/ml DMSO. Only compounds **1a** and **1e** displayed activity in this screening, and they were studied further using four different concentrations (5, 10, 20, and 40 mg/ml) and employing a modified method [Method II in the paper of Elo (2007)].

Micro-scale determination of MIC

MIC values were determined using a micro-scale modification of our previously published MIC method (Elo *et al.*, 2007). In this micro-scale method, the measurements were carried out using 96-well deep-well plates made of non-transparent polypropylene (Nunc, Roskilde, Denmark; cat. no. 278752). In these round-well plates, the volume of

each well was 2 ml. Detection of microbial growth was based on visual inspection of the wells of these non-transparent plates. The plates were sterilized by autoclaving at 121 °C for 15 min. During incubations, the plates were covered by silicone sealing mats that allow diffusion of oxygen to the wells (AxyMat; Axygen Scientific Inc, Union City, CA, USA; cat. no. AM-2ML-RD).

In our method, the plates and mats were recycled. After each use, the plates were first soaked in an 1% solution of Virkon® S (DuPont Animal Health Solutions Europe, Sudbury, Suffolk, UK) for at least 15–20 min, and the wells were cleaned with a bottle brush equipped with a cotton tip. Then, the plates and mats were washed in a laboratory washing machine, allowed to dry, and finally sterilized.

A suspension of the appropriate microbial strain was prepared in the appropriate sterile broth using colonies from a fresh pure culture on agar. In preparing these suspensions, a McFarland standard series (McFarland Standard Set, cat. no. SD 2350; Pro-Lab Diagnostics, Richmond Hill, ON, Canada) was employed. In the case of the cocci, the rods, and the yeasts, respectively, the goal was a 0.5 McFarland, a 2 McFarland, and a 4 McFarland suspension, respectively. (Initially, for rods, the goal was an 1 McFarland suspension, but during the work, it turned out that a 2 McFarland suspension is preferred.) These turbidities were chosen because they correspond to roughly $0.5 - 2 \cdot 10^8$ colony forming units per ml. In each case, the above suspension was diluted 40-fold with the broth. Below, this diluted suspension is called the inoculum suspension.

The MIC measurements were carried out as follows: 1 ml of the appropriate sterile broth was pipetted into each well of a deep-well plate, followed by 170 μl of the thoroughly mixed inoculum suspension (except for those wells that were used as sterile controls). Into each well, 30 μl of a DMSO solution of the test compound was then added, using 12 different concentrations ranging from 0.005 to 40 mg/ml DMSO (two-fold dilutions, *i.e.*, 40, 20, 10 etc. mg/ml). These concentrations are equivalent with 0.125 to 1000 μg/ml of the test compound in the final culture. In every instance, pipetting was carried out avoiding splashing or creation of bubbles.

Controls into which only DMSO was added, as well as controls with no added chemicals, were also included in each case, and invariably displayed growth, while the sterile controls (sterile broth with and without DMSO) never displayed growth. For each control type, four wells were included in each measurement series.

Positive (*i.e.*, antibiotic) controls were always employed. Thus, in the case of *B. cereus* and *P. aeruginosa*, 30 μl of a sterile aqueous solution of doxycycline were added into a well, using 12 different concentrations of the antibiotic in the aqueous solution (0.0012 to 20 mg/ml; 4-fold concentration differences between 0.0012 and 0.078 mg/ml, 2-fold concentration differences between 0.078 and 20 mg/ml), resulting in concentrations ranging from 0.03 to 500 μg/ml in the final culture. In the case of *E. coli*, *S. aureus*, and *S. lactis*, 12 different concentrations (0.0012 to 5.0 mg/ml; 2-fold concentration differences between 0.005 and 5.0 mg/ml, 4-fold difference between 0.0012 and 0.005 mg/ml) of sterile aqueous cefuroxime were analogously added to the cultures, resulting in final concentrations ranging from 0.03 to 125 μg/ml. In the case of yeasts, 12 different concentrations (0.0010 to 8.0 mg/ml; 4-fold concentration differences between 0.0010 and 0.015 mg/ml, 2-fold concentration differences between 0.015 and 8.0 mg/ml) of sterile aqueous solutions of amphotericin B were analogously added to the cultures resulting in final concentrations of 0.024 to 200 μg/ml.

All measurements on test compounds and antibiotic controls were performed in duplicate. After the pipetting procedures, the plates were gently shaken for 5 min using a Delfia Plateshake shaker (Wallac Oy/PerkinElmer, Turku, Finland) and were then incubated at the appropriate growth temperature for 44 h. The wells were studied for visible growth at 24 h and 44 h.

Determination of MBC and MFC

The MBC and MFC values, *i.e.* the minimal bactericidal and fungicidal concentrations, of the test compounds were determined using a modification of the approach described by Hacek *et al.* (1999). The details of our procedure were as follows.

Determination of microbial count after treatment with a test substance or antibiotic control

Samples were taken at 24 h from those wells of the MIC test micro-titration plate, into which either a test substance or a control antibiotic had

been pipetted and in which no growth could be visually observed (*i.e.*, from wells that on the basis of visual inspection were found to contain a clear liquid). Before drawing a sample, the contents of the well were thoroughly mixed by 'pumping' 20 times with the aid of a pipette. From each well, one 100-μl sample was removed with the aid of the pipette and was streaked across an agar plate (diameter 90 mm) so that a straight line was formed across the plate. The inoculum was allowed to absorb into the agar for 25 min (so that the test substance or control antibiotic was absorbed into a narrow linear portion of the agar plate, the rest of the plate being essentially free of the compound), after which the microbes were spread outwards from the streak line over the entire surface of the agar plate with the aid of a plastic angle rod ('Servant'; Konstrumed Oy, Tampere, Finland). This 'streak' technique was adopted from the method of Shanholtzer *et al.* (1984) and has been used also by Hacek *et al.* (1999). For formation of colonies, the agar plates were incubated for 24 h (in the case of bacteria) or 44 h (in the case of fungi) at +30 °C or +37 °C, as appropriate for the microbe in question.

Determination of initial microbial count

At the beginning of the MIC test, 515 μl of sterile broth and 85 μl of the thoroughly mixed inoculum suspension were pipetted into a sterile test tube. The resultant suspension thus contained an equal number of microbes per unit of volume as did the wells on the micro-titration plates at the beginning of the MIC test. This suspension was diluted with sterile broth (1:100 and 1:1000). This procedure was performed in duplicate. Then, an 100-μl sample of each of the 1:100 and 1:1000 dilutions was pipetted onto a 90-mm agar plate, as a streak across the diameter of the plate, the aim of this 'streak' procedure being to imitate the procedure performed in the determination of microbial counts after treatment with test substances and control antibiotics. The liquid of the inoculum was allowed to absorb into the agar plate for 25 min, after which the microbes were spread over the entire surface of the plate with the aid of a plastic angle rod. The plates were incubated as above.

Determination of MBC and MFC values. Calculations performed

After the incubation, colonies on the agar plates were counted. According to common practice in clinical microbiology (see Pearson *et al.*, 1980), we define MBC and MFC as the lowest concentration of the test compound that kills 99.9% of the bacteria or fungi, respectively. When the number of microbes in the untreated samples taken just before the start of the MIC test, as well as those in the samples taken from the wells after the test, have been determined with the aid of the colony counts, the lowest tested concentration of the test substance capable of killing at least 99.9% of the microbes can be determined. Thus, we compared the number of living, colony-forming microbes in the treated samples with a 'cut-off end-point number' corresponding to the survival of 0.1% of the colony-forming microbes that were present at the beginning of the MIC test. The cut-off end-point number is thus 1/1000 (= 0.1%) of the colony count from the pre-MIC test samples, and according to common practice, we use the symbol n for it. Instead of using n as the cut-off end-point number, Anhalt *et al.* (1980) have used a parameter called N that is intended to give a 95% confidence limit. They calculated N as follows: $N = n + 2\sqrt{n}$. We calculated values of MBC and MFC both by using n and by using N as the cut-off end-point number. Also $N' = n - 2\sqrt{n}$ (an 'analogue' of N) was calculated in each case.

Results

Development of a simple micro-scale method for the determination of MIC values

We describe a novel method for determining MIC values that is easier to perform and to automate than are classical macro-scale test-tube methods. For this method, much less incubator space is required than for classical methods and much less materials are consumed. In a classical method, the equivalent of each deep-well microtitration plate would be a total of 96 large test tubes. The visual inspection of the wells is rapid and simple.

Instead of DMSO, also other solvents tolerated by the microbes in question can be used, including aqueous systems such as sterile water that was used in the case of the control antibiotics and worked well.

Development of a method for the determination of MBC and MFC values

It is common practice in clinical microbiology to define MBC and MFC as the lowest concentration of the test compound that kills 99.9% of the bacteria or fungi, respectively (see Pearson *et al.*, 1980; Anhalt *et al.*, 1980; Peterson and Shanholtzer, 1992), and we adhered to that definition in the present study. Further, we intended to perform the measurements in a setting where the turbidity of the microbial suspension and thus, roughly, also the number of microbes at the beginning of the test is standardized.

Further, we wanted to develop a simple method for determining MBC and MFC values without the need to centrifuge and wash the microbes for removal of the antimicrobial agent to be tested. Methods involving centrifugation are tedious and labour-intensive, and they would be expected to be prone to errors because of incomplete recovery of the microbes and possible lethal effects of the centrifugations and rapid changes of the milieu during the washing procedures, and might also be complicated by incomplete removal of the test substance if it is present as a suspension either because of limited solubility in DMSO (or other suitable solvents) or because of precipitation in the aqueous test system.

In the 1980's, Shanholtzer *et al.* (1984) studied a variety of macro-dilution and micro-dilution techniques for MBC testing of five clinically important antibiotics with clinical isolates of *S. aureus*. In that study, standard micro-dilution failed to give reproducible MBC results, even when a strictly defined protocol was used. For micro-dilution tests, they used, among others, a technique in which frozen broth micro-dilution Micro Scan trays were employed. The present method is technically much simpler than the micro-dilution methods of Shanholtzer *et al.* (1984), and no special and difficult-to-obtain equipment such as special frozen trays is needed.

It is well known that so-called 'skip tubes' (or, in micro-methods, analogously 'skip wells' etc.) may occur (Peterson and Shanholtzer, 1992; Anhalt *et al.*, 1980). This term relates to a phenomenon in which one or more tubes or wells in a series of broth dilutions in MIC tests appear to be free of microbial growth or, in MLC tests, contain less than 0.1% of colony-forming (live) microbes as compared to the initial inoculum, whereas one or more tubes or wells containing higher concentrations of the antimicrobial agent show visible growth or contain more than 0.1% of colony-forming units as compared to the initial inoculum. In our method, we define the MBC or MFC value as the lowest concentration of the test substance, in the presence of which the number of colonies is less than the cut-off value and will remain so for all higher concentrations of the drug.

Antimicrobial activity of formylchromones

We studied, using a panel of seven microbial species, the antimicrobial effects of several formylchromones, **1a**–**e**. These aldehydes were chosen for study in part because we have for a long time been interested in the highly potent antimicrobial activity of aldehydes, most notably aromatic aldehydes of the salicylaldehyde (2-hydroxybenzaldehyde) type, whose antimicrobial properties we have studied in detail (Pelttari *et al.*, 2007a, 2011). In part, these compounds were chosen for study because they carry some structural similarity with well-known antibiotics, namely nalidixic acid and fluoroquinolones.

We first studied the properties of the formylchromones **1a**–**e** (Fig. 1) with the aid of agar diffusion. Two compounds, 3-formylchromone (**1a**) and 3-formyl-6-nitrochromone (**1e**), displayed potent antimicrobial activity, while the rest of the compounds did not display any activity (see Tables I and II). 3-Formylchromone was highly active against both of the yeasts and all of the bacterial strains studied, except for *P. aeruginosa*. In most cases, it had high activity even at the lowest concentration studied. Its 6-nitro-substituted analogue **1e** was active against all microbes studied, displaying highly potent activity against *S. cerevisiae* but having less potent activity than the non-nitro-substituted congener **1a** against *B. cereus*, *C. albicans*, *E. coli*, and *S. lactis*, and displaying weak but distinct activity against *S. aureus* and *P. aeruginosa*. The inactivity of the remaining compounds may either be an intrinsic property of the compounds or may be due to the fact that they were essentially insoluble in DMSO and other suitable solvents tested and thus had to be used as suspensions.

We further determined the MIC, MBC, and MFC values of compounds **1a** – **e** (Tables III and IV). Compounds **1a** and **1e** that displayed activity in the agar diffusion test were found to be active

Table I. The results of paper disc agar diffusion studies on 3-formylchromone (**1a**). The diameter of the disc was 6 mm. The tests were performed as described in Method II of Elo (2007). In each case, the diameter given represents the mean of results of three or four individual measurements (individual paper discs). Control antibiotics gave the following inhibitory zones ± S.D., as based on results of 15–20 individual measurements in each case: doxycycline, (35 ± 1) mm for *B. cereus* and (16 ± 1) mm for *P. aeruginosa*; cefuroxime, (28 ± 1) mm for *E. coli*, (34 ± 1) mm for *S. aureus*, and (33 ± 1) mm for *S. lactis*; and amphotericin B, (18 ± 1) mm for *C. albicans* and (13 ± 2) mm for *S. cerevisiae*. All S.D. values are rounded to the closest whole number in mm.

Microbe	Inhibitory zone ± S.D. [mm]			
	40 mg/ml	20 mg/ml	10 mg/ml	5 mg/ml
B. cereus	30 ± 2	25 ± 1	22 ± 1	18 ± 1
E. coli	28 ± 1	28 ± 1	24 ± 2	19 ± 1
P. aeruginosa	7 ± 1	7 ± 1	7 ± 0	7 ± 1
S. aureus	21 ± 1	16 ± 1	13 ± 1	10 ± 1
S. lactis	19 ± 1	15 ± 1	10 ± 1	7 ± 1
C. albicans	26 ± 1	26 ± 1	23 ± 1	19 ± 1
S. cerevisiae	34 ± 2	35 ± 1	28 ± 2	21 ± 1

Table II. The results of paper disc agar diffusion studies on 3-formyl-6-nitrochromone (**1e**). The diameter of the disc was 6 mm. The tests were performed as described in Method II of Elo (2007). In each case, the diameter given represents the mean of results of three or four individual measurements (individual paper discs). For results on control antibiotics, see Table I. All S.D. values are rounded to the closest whole number in mm.

Microbe	Inhibitory zone ± S.D. [mm]			
	40 mg/ml	20 mg/ml	10 mg/ml	5 mg/ml
B. cereus	18 ± 1	16 ± 1	13 ± 1	9 ± 0
E. coli	14 ± 1	13 ± 2	10 ± 1	8 ± 0
P. aeruginosa	8 ± 0	8 ± 0	7 ± 0	7 ± 0
S. aureus	8 ± 1	8 ± 1	7 ± 0	6 ± 1
S. lactis	13 ± 1	9 ± 1	7 ± 1	7 ± 1
C. albicans	13 ± 0	11 ± 0	9 ± 0	9 ± 0
S. cerevisiae	35 ± 0	33 ± 1	30 ± 1	26 ± 0

also in the MIC test, while the remaining compounds displayed no activity in the concentration range studied. Both active compounds displayed activity against all microbes studied except *P. aeruginosa*. Thus a distinct correlation between the results of the agar diffusion test and those of the MIC test was observed. Both compounds had noteworthy activity against the yeasts studied, and 3-formylchromone (**1a**) also against *B. cereus*. In the MBC and MFC tests, however, the 6-nitro-substituted congener **1e** displayed activity only against one microbe, *E. coli*, at 1000 μg/ml and no activity against the other species tested. Compound **1a** was able to kill the yeasts and *E. coli* in concentrations around or below 250 μg/ml but was inactive or essentially inactive against all other microbes tested, including *B. cereus*.

Discussion

The antimicrobial activity of the formylchromones **1a** and **1e** suggests that further studies on related compounds might be worthwhile. The antimicrobial activity of this class of compounds has been previously studied by El-Shaaer *et al.* (1998) who found some activity but, unfortunately, their report does not reveal the structures that correspond to the individual compounds tested. Whether formylchromones and fluoroquinolone antibiotics share a common mechanism of action remains to be studied.

In determining MBC and MFC values, the microbes are treated with the antimicrobial agent in liquid culture, and the number of microbes surviving the treatment is usually determined by plate counting. If the antimicrobial agent is carried over the plates, it may prevent the formation of colonies in spite of not having killed the microbes, resulting in wrong results, even bacteriostatic and fungistatic compounds appearing as effective bactericidal and fungicidal agents, respectively. 'Real' bactericidal and fungicidal substances, if applied with the microbes onto the plates, may also give rise to erroneously low MBC and MFC values. Removal of the antimicrobial agent from the liquid culture by the aid of centrifugation is labour-intensive. Further, microbes may be killed or loose their colony-forming ability during the washing procedures. Compounds forming a precipitate in the liquid culture are not infrequent in drug discovery and cannot be removed by centrifugation. Several investigators have used an alternative method, namely application of the liquid from MIC tests in the form of a streak on agar (Shanholtzer *et al.*, 1984; Hacek *et al.*, 1999). We applied that simple technique with success. It might be claimed that there is no guarantee that the microbes are effectively spread from the area of the streak to the whole plate, which would lead to false results, the antimicrobial agent being able to prevent microbial growth and formation of colonies on agar even in the case of wells, in which its concentration was not lethal to the microbes. Our results, however, indicate that this risk can be

Table III. The results of the MIC measurements performed.

Compound	MIC [μg/ml]													
	B. cereus		*E. coli*		*P. aeruginosa*		*S. aureus*		*S. lactis*		*C. albicans*		*S. cerevisiae*	
	24 h	44 h	24 h	44 h	24 h	44 h	24 h	44 h	24 h	44 h	24 h	44 h	24 h	44 h
1a	63	63	250	250	>1000	>1000	250	250	750[a]	750[a]	63	94[a]	47[a]	94[a]
1b	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
1c	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
1d	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
1e	750[a]	750[a]	250	375[a]	>1000	>1000	?[b] (≥500)	500	750[a]	750[a,b]	16	190[a,b]	31	63
Doxycycline	0.13	0.16[a]			63	125					0.78	0.78	6.3	6.3
Amphotericin B														
Cefuroxime			2.0	2.0			2.0	2.0	0.50	0.50				

[a] Mean of two determinations (the results of the two determinations differed by one dilution).
[b] MIC value difficult to determine because of precipitation of compound in the medium.

Table IV. The results of MBC and MFC tests on 3-formylchromone (**1a**) and 3-formyl-6-nitrochromone (**1e**). Samples for plate counts were taken from the MIC test wells at 24 h. Colonies were counted after 1 day (bacteria) or 2 days (yeasts).

Microbe	MBC or MFC [μg/ml]				
	1a	**1e**	Doxycycline	Cefuroxime	Amphotericin B
B. cereus	> 1000	> 1000	94[a]		
E. coli	250	1000		2.0	
P. aeruginosa	> 1000	> 1000	125		
S. aureus	1000	> 1000		2.0	
S. lactis	> 1000	> 1000		0.50	
C. albicans	190[a]	> 1000			1.6
S. cerevisiae	190[a,b]	> 1000			13

[a] Mean of two determinations (the results of the two determinations differed by one dilution).
[b] If *n* or *N* is used as the cut-off end-point number, the MFC value is 190, but if *N'* is used, the MFC value is 250. In all other cases, all three parameters give exactly the same MBC or MFC value.

excluded, since we used the streak technique also in determining the initial microbial counts, and in those cases, no antimicrobial agent was present in the liquid culture. If, in those cases, the microbes would have remained at the area of the streak and its vicinity, this would have been noticed easily; yet it never happened.

In our method, the standardization of the number of microbes in the inoculum suspension was successfully carried out with the aid of the McFarland standard set. Thus, in the case of each microbial species, the number of colony-forming units per ml was roughly similar in separate experiments. This intra-species standardization is of significance, especially if comparison of the effects of different drug candidates constitutes the goal, inter-species standardization being not of similar significance. If, however, the goal is to compare drug effects on different types of microbes, then inter-species standardization (that is more difficult to perform successfully) becomes highly important. In our study, it turned out that the number of rods in the initial inoculum tended to be somewhat lower than that of the other microbial species, and the use of a 2 McFarland standard is to be preferred as compared to the 1 McFarland one that was initially used.

On the basis of the results obtained on formylchromones, on one hand, it appears that it might be worth adding MIC tests to early drug screen-

ing panels since agar diffusion testing alone may give falsely promising results in some cases. On the other hand, some active lead compounds that may constitute a good starting point for drug development might be missed if only MIC tests were used in early screening.

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